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Identification of Novel Crk-associated Substrate (p130^{Cas}) Variants with Functionally Distinct Focal Adhesion Kinase Binding Activities*

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Background: The tumor promoter p130^{Cas} utilizes its SH3 domain to control cell adhesion and migration.
Results: We identified N-terminal extended p130^{Cas} variants with enhanced SH3 domain binding activity which modulates cell migration and invasion.
Conclusion: Naturally occurring p130^{Cas} variants exhibit differential biological activities.
Significance: Knowledge on these variants may explain how p130^{Cas} controls cellular programs and drives carcinogenesis.

Elevated levels of p130^{Cas} (Crk-associated substrate)/*BCAR1* (breast cancer antiestrogen resistance 1 gene) are associated with aggressiveness of breast tumors. Following phosphorylation of its substrate domain, p130^{Cas} promotes the integration of protein complexes involved in multiple signaling pathways and mediates cell proliferation, adhesion, and migration. In addition to the known *BCAR1*-1A (wild-type) and 1C variants, we identified four novel *BCAR1* mRNA variants, generated by alternative first exon usage (1B, 1B1, 1D, and 1E). Exons 1A and 1C encode for four amino acids (aa), whereas 1D and 1E encode for 22 aa and 1B1 encodes for 50 aa. Exon 1B is non-coding, resulting in a truncated p130^{Cas} protein (Cas1B). *BCAR1*-1A, 1B1, and variant 1C mRNAs were ubiquitously expressed in cell lines and a survey of human tissues, whereas 1B, 1D, and 1E expression was more restricted. Reconstitution of all isoforms except for 1B in p130^{Cas}-deficient murine fibroblasts induced lamellipodia formation and membrane ruffling, which was unrelated to the substrate domain phosphorylation status. The longer isoforms exhibited increased binding to focal adhesion kinase (FAK), a molecule important for migration and adhesion. The shorter 1B isoform exhibited diminished FAK binding activity and significantly reduced migration and invasion. In contrast, the longest variant 1B1 established the most efficient FAK binding and greatly enhanced migration. Our results indicate that the p130^{Cas} exon 1 variants display altered functional properties. The truncated variant 1B and the longer isoform 1B1 may contribute to the diverse effects of p130^{Cas} on cell biology and therefore will be the target of future studies.

p130 Crk-associated substrate (p130^{Cas})[3] is an adapter protein that functions as a scaffold integrating large multi-protein complexes in response to stimuli such as growth factor and hormones and integrin engagement (1–3). Through its interaction with Src family members, focal adhesion kinase (FAK) and PI3K, it recruits the adaptor proteins Crk and Nck to its phosphorylated SD. These complexes regulate cell survival, cell cycle regulation, proliferation, adhesion, migration, and invasion (1, 3, 4). These cellular programs are frequently deregulated in various malignancies (5–7). For example, p130^{Cas} is associated with the development and progression of pulmonary disease (8), cardiovascular disease (9), and particularly cancer (1, 4, 10, 11). Increased p130^{Cas} protein expression promotes tumor progression and aggressiveness and mediates resistance to the anti-tumor drugs adriamycin and tamoxifen (12, 13). Its gene was independently identified as breast cancer antiestrogen resistance 1 gene (*BCAR1*) (14). Here, we refer to the gene and mRNA as *BCAR1* and to the protein as p130^{Cas}.

Because several studies link p130^{Cas} to important cellular programs and diseases, our goal is to understand how the complex p130^{Cas} biology is regulated. Studies of mouse p130^{Cas} (15) and our recent investigation on human p130^{Cas} (10) identified two p130^{Cas} variants encoded by alternative first exons of *BCAR1*. This is of special interest because the amino acids (aa) encoded by exon 1 directly flank the p130^{Cas} SH3 domain. Different N-terminal extensions may influence the interactions of the SH3 domain with other proteins and thus the function of p130^{Cas}. Of note, the p130^{Cas} SH3 domain and its interaction with FAK are necessary for the phosphorylation and translocation of p130^{Cas} to focal adhesions (FAs) (16). There it controls FA turnover and adhesion and promotes the migratory/invasive response of the cell to certain stimuli (17–19). Therefore, we were interested in whether additional exon 1 p130^{Cas} variants with distinct N-terminal extensions and altered functions exist.

In this study, we identified four novel *BCAR1* mRNAs generated by alternative first exon usage and splicing. The resulting N-terminal protein variants display differences in their binding

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[3] The abbreviations used are: p130^{Cas}, p130 Crk-associated substrate; aa, amino acids; ER, estrogen receptor; ev, empty vector; FA, focal adhesion; FAK, focal adhesion kinase; IB, immunoblotting; IP, immunoprecipitation; NT, N-terminal; SD, substrate domain; SH, Src homology; WCE, whole cell extracts; EST, expressed sequence tag; abs, antibodies.

TABLE 1
Primer sequences

Name	Forward (5′–3′)	Name	Reverse (5′–3′)
NT-1A-EcoRI	CCGGAATTCCCGGACACCATGAACCACC	Reverse-V5 XhoI	CCGCTCGAGTGTGGGGCATGGAGGCCAGGC
NT-1B1-EcoRI	ATAGAATTCATGCCTGCCAAGC		
NT-1C- EcoRI	CCGGAATTCGCGGCCAAGATGTCCGTGCC		
NT-1D-I	CCCTGGTCCTTCCTTTGAGTGGAACGTGCTGGCC		
NT-1D-II	AGGAAGCAGAACAGAGGGGTCGGACCCCTGGTC		
NT-1D-III- EcoRI	ATAGAATTCATGCTCACCCACCGTCCCCAGGAAGC		
NT-1E-EcoRI	CCGGAATTCGAGGGCATGCACTGCCCTGG		
NT-1Δ-EcoRI	CCGGAATTCATGAACGTGCTGGCCAAAGCG		
Cas1A-f	GCGGCCGCGGCCCGCCGGACACCATGA	Cas1A-r	GTTAACAACTGCACTGGCCCTGTCAGG
Cas1B-f-I	AGGGGAAGCAGGAACGTGCTGGCC	Cas1B-r-NotI	ATAGCGGCCGCCCCTCAGGCGGCTGCCAGC
Cas1B-f-II	AGAAGGGGATGGGAGGGGAAGCAGG		
Cas1B-f-III- EcoRI	ATAGAATTCATGCTTCAGAAGGGGATGGGA		
Cas1C-f	GCGGCCGCGCGGCCAAGATGTCCGTGC	Cas1C-r	GTTAACTCTCCTGGTAAGGCGGAGG
Cas1E-f-EcoRI	CGGAATTCAGTCAGGAGAGGGCATGCACTGC	Cas1E-r-EcoRI	CGGAATTCACCCTCAGGCGGCTGCCAGC
Cas1Δ-f-EcoRI	CCGGAATTCATGAACGTGCTGGCCAAAGCG	Cas1Δ-r-EcoRI	CCGGAATTCTCTCCTGGTAAGGCGGAGG
BCAR1–1Aq	GACACCATGAACCACCTGAA	BCAR1q-rev	TCATACATGCCCACCAAGAT
BCAR1–1Bq	GCTTTCATGTGCTGAGGAGGC	BCAR1q-rev	
BCAR1–1B1q	TGCTGGAGGGCCGAACGTG	BCAR1q-rev	
BCAR1–1Cq	CGGCCAAGATGTCCGTGC	BCAR1q-rev	
BCAR1–1Dq	CTTCCTTTGAGTGGAACGTGC	BCAR1q-rev	
BCAR1–1Eq	GGCCCACACCCAAGGATCC	BCAR1q-rev	
GAPDHq-f	TGCACCACCAACTGCTTAGC	GAPDHq-r	GGCATGGACTGTGGTCATGAG

to FAK. Overexpression in a $p130^{Cas}$-null background leads to altered cell morphology and differential migratory and invasive potential. Importantly, the naturally occurring truncated variant and the longest isoform exhibited greatly diminished and significantly increased functionality, respectively.

EXPERIMENTAL PROCEDURES

Generation of Expression Constructs

I.M.A.G.E. clones for human full-length $p130^{Cas}$ cDNAs including exon 1A (Cas1A), Cas1C, and Cas1E ID 6428300, ID 6428300, and ID 4940045, respectively, were obtained from Open Biosystems. All expression constructs for N-terminal $p130^{Cas}$ variants were generated by PCR using *Pfu* polymerase (Stratagene) and subcloned into pcDNA4/V5-HisA vector (Invitrogen).

Expression constructs for full-length $p130^{Cas}$ were generated by PCR and subcloned into the retroviral expression vector pCX_{bsr} (20). Primer sequences are provided in Table 1.

The coding exon 1B1 sequence (bp 285–434) flanked by an EcoRI and NarI site was ordered from GenScript. The EcoRI/NarI-digested exon 1B1 cDNA and the NarI/NotI-digested 3′ $p130^{Cas}$ region (2.5 kb) were ligated into pCX_{bsr} (EcoRI/NotI) resulting in full-length Cas1B1. The FAK construct has been described (20). Constructs were validated by sequencing. The Qiagen Plasmid Maxi Kit was used for DNA preparation.

Database Analysis

BCAR1 and the exon 1 mRNA and protein variants were analyzed using database resources and software from AceView (21), the European Molecular Biology Laboratory (EMBL)-European Bioinformatics Institute (EBI), Genomatix (22), and the National Center for Biotechnology Information (NCBI) (23). Expressed sequence tags (ESTs) and cap analysis of gene expression (CAGE) tags for each variant were identified at AceView, Genomatix (ElDorado), and NCBI (BLAST, EST, and UniGene) databases. To further support the existence of the variants, the genes, potential promoters, completeness of transcripts, and proteins in different species were analyzed with software and databases at Genomatix (Gene2Promoter, MatInspector, and ElDorado) and NCBI (Nucleotide and Protein). Putative functional protein motifs in the N-terminal part of each variant up to the end of the SH3 domain were identified using the ELM server (24) and Scansite 2.0 (25).

Tissue Samples, Cell Lines, and Culture Conditions

Tissue Samples—Historical human tissue samples from normal breast, breast carcinoma, normal colon, colon carcinoma, stomach, liver, kidney, and rectum were used.

Cell Lines—Cell lines were purchased from the American Type Culture Collection (ATCC) or obtained as indicated. The following cell lines were used: human foreskin fibroblast, BJ1; untransformed mammary epithelium, MCF-10A; human breast carcinoma, estrogen receptor (ER)-negative, BT-20 (Sam Lee, Massachusetts General Hospital); human breast carcinoma, ER-positive, MCF-7, T47D; human embryonic kidney epithelial cells 293T (293T); and $p130^{Cas}$-deficient murine fibroblasts ($Cas^{-/-}$) (26).

Culture Conditions—For $Cas^{-/-}$ cells, the culture conditions were: DMEM, 5% calf serum. For MCF-7 cells, the culture conditions were: RPMI 1640, 5% FBS, and 4 mM glutamine. For all other cell lines, the culture conditions were: DMEM, 5% FBS. Media were supplemented with antibiotics/sodium pyruvate, and the cells were maintained as described (10, 27). Sodium vanadate treatment was performed at 500 μM for 6 h prior to cell harvesting to accumulate phosphorylated $p130^{Cas}$.

Real Time RT-PCR

RNA was extracted using RNeasy kit including the DNase treatment step (Qiagen), and RT-PCR was performed as described (10, 27, 28). cDNA was treated with RNase H (Life Technologies). Real time PCR was performed in 20-μl reactions in triplicate using ABI PRISM 7900HT sequence detection sys-

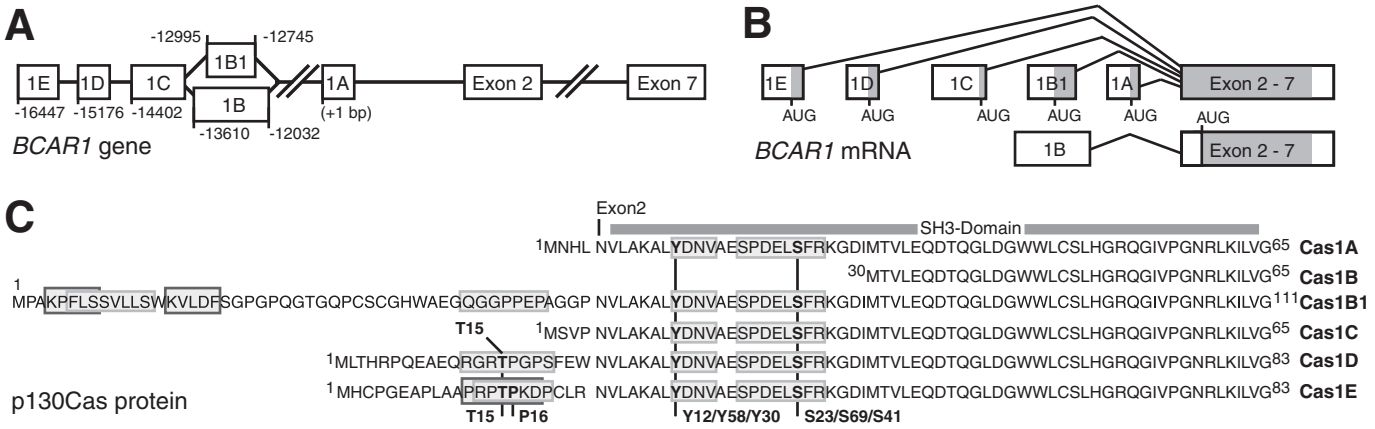

FIGURE 1. ***BCAR1* has multiple first exons.** *A*, schematic of *BCAR1*. //, sequences of exons 3 through 6 and between exons 1A and 1B were omitted. Numbering indicates the transcription start site of each variant relative to exon 1A (+1). *B*, *BCAR1* exon 1 mRNA variants. The translation start site (AUG) for each variant is indicated. *Gray boxes*, translated regions. *C*, protein sequences and putative motifs in the N-terminal region of the p130^{Cas} isoforms. Shown are the N termini including the complete SH3 domain of each variant and putative phosphorylation and interaction sites. Cas1B is numbered relative to Cas1A.

tem version 2.3 (Applied Biosystems) and 2× FastStart Universal SYBR Green PCR Master (ROX) (Roche Applied Science) using 10 ng of cDNA. Exon-overlapping PCR primers were applied, and the sequences are presented in Table 1. PCR specificity and amplicon size were validated by electrophoresis through a 2% agarose gel and subsequent sequencing. Efficiency of each PCR was >98% as determined by performing the PCRs for each primer pair with serial DNA dilutions. Comparative Ct method was applied for relative quantification of expression of each gene between samples (29) and normalized to GAPDH.

Retroviral Transduction and Transient Transfection

Retroviral transduction and transient transfection were carried out as described previously (10, 11, 30).

Protein Analysis and Immunoprecipitation

Whole cell extracts were prepared and analyzed by immunoblotting as described (31). Some proteins were separated on low-bis (ratio 149:1) SDS-polyacrylamide gels as indicated. For immunoprecipitation (IP) of FAK, 293T cells transiently transfected with FAK-pcDNA3 or p130^{Cas} N-terminal (NT) constructs in pcDNA4-V5 or Cas$^{-/-}$ stable p130^{Cas} variant transductants were lysed in HNTG (50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM EDTA) buffer. Lysates containing ectopically expressed FAK were mixed with lysates expressing p130^{Cas} NT or full-length p130^{Cas} and incubated at 4 °C for 2 h prior to incubation with V5 (1 μg) or p130^{Cas} (2 μg) antibodies (abs) at 4 °C overnight. Washed GammaBind^{TM} G-Sepharose^{TM} beads (GE Healthcare) were added to the samples for an additional 90 min at 4 °C. Precipitates were washed four times with ice-cold HNTG buffer (0.1% Triton X-100) and then subjected to immunoblotting. The following antibodies were used: mAbs, β-actin (Sigma), FAK (Clone 77, BD Transduction Laboratories), p130^{Cas} (immunoblotting, clone 21, BD, Transduction Laboratories), and V5 (Invitrogen); polyclonal rabbit abs, p130^{Cas} (IP, C-20, sc-860, Santa Cruz Biotechnology); phosphospecific anti-p130^{Cas} tyrosines, 165 (4015), 249 (4014), and 410 (4011) (Cell Signaling).

Migration and Invasion Assays

Cas variant cells (migration, 7.5 × 10^4; invasion, 5 × 10^4) were layered in the upper compartments of a Transwell (migration) chamber or invasion chambers (both chambers: 6.5-mm-diameter polycarbonate filter; 8-μm pore size; Costar) and incubated at 37 °C for 6 h (migration) or 22 h (invasion). Culture medium (20% calf serum) was added to the lower compartment. Migration and invasion of the cells to the underside of the filter were evaluated by staining with crystal violet and quantified by counting the cells in three random fields per experiment. Three independent assays were performed in triplicate.

Phosphatase Treatment

Cells were washed twice with 1× PBS, lysed in PBS/0.5% SDS, and incubated at 95 °C for 10 min. Cell lysates (60 μg) were treated with 20 units of calf intestinal phosphatase (New England Biolabs) or water control in New England Biolabs buffer 3 for 1 h at 37 °C and subjected to immunoblotting as described.

RESULTS

BCAR1 Has Multiple First Exons—We recently described two *BCAR1* exon 1 variants (*BCAR1*-1 and *BCAR1*-1′, which we refer to here as Cas1A and Cas1C, respectively) (10). Based on these findings, we investigated whether additional p130^{Cas} variants with different functions exist. Subsequent comprehensive database analyses revealed that *BCAR1* contains five additional exons upstream of exon 1 (1A, first identified WT, RefSeq NM_014567.3) (Fig. 1). ESTs and cap analysis of gene expression tag databases suggested that these exons represent alternative first exons, hence designated by their genomic position related to 1A as exons 1B (XM_005256260.2), 1B1 (NM_001170714.1), 1C (NM_001170718), 1D (NM_001170716.1), and 1E (NM_001170715.1). Exons 1A and 1C encode for four aa, whereas 1D and 1E translate into 22 aa (Fig. 1*C*). Exon 1B1 encodes for 50 aa. Exon 1B is non-coding, leading to a truncated form of the p130^{Cas} protein (Cas1B) starting at aa 30 in reference to Cas1A, thereby lacking 24 aa of the SH3 domain (aa 6–64). The variant proteins 1A and 1C are evolutionarily conserved in mammals, whereas the longer variants 1B1, 1D, and

TABLE 2

Database analysis of p130Cas exon 1 variant proteins

Bold and underlined letters indicate amino acid changes compared with *Homo sapiens*.

Species	Cas1A	Cas1B	Cas1C	Cas1D	Cas1E
Homo sapiens	M NHL	Protein starts at aa 30 in exon 2	M SVP	MLTHRPQEAEQ RGRTPGPSFEW	MHCPGEAPLAA PRPTPKDPCLR
Pan troglodytes	M NHL	Protein starts at aa 30 in exon 2	M SVP	MLTHRPQEAEQ RGRTPGPSFEW	MHCPGEAPLAA PRPTPKDPCLR
Macaca mulatta	M NHL	Not found	Not found	MLTHRPQ**A**AEQRG**Q**TP**A**PSF**G**W	MHCPGEAPLAA PRPTPKDPCLR
Physeter catodon	M N**Y**L	Protein starts at aa 30 in exon 2	M SVP	Not found	Not found
Bos taurus	M N**Y**L	Not found	M SVP	Not found	Not found
Rattus norvegicus	M **KY**L	Not found	M **T**VP	Not found	Not found
Mus musculus	M **KY**L	Not found	M **T**VP	Not found	Not found

1E were only identified in primates (Tables 2 and 3). The truncated 1B isoform was detected in Primates (Hominidae only) and Cetacea. Once complete genetic information of lower mammalian species or other classes is available, the variants may also be identified in additional species. Motif analysis of the NT region of $p130^{Cas}$ revealed that Cas1A and 1C and the novel variant proteins Cas1B1, 1D, and 1E contain a YDNV motif representing a putative phosphorylation site (Tyr-12/58/30) at aa 12–15, 58–61, and 30–33, respectively, and a SPDELSFR motif at aa 18–25/64–71/36–43 predicted for phosphorylation at Ser-23/69/41. In addition, the longer variants Cas1D and Cas1E comprise a predicted phosphorylation site at Thr-15. The longest variant Cas1B1 contains additional potential interaction motifs at aa 4–8, 6–13, 15–19, and 40–46. Therefore, *BCAR1* has six first exons resulting in protein isoforms that possibly possess altered functions.

BCAR1 Exon 1 Variants Are Expressed in Human Cell Lines and Tissues—To reveal the expression pattern of the *BCAR1* exon 1 variants, real time RT-PCR was performed on mRNA isolated from immortalized mammary epithelial (MCF-10A), estrogen receptor-positive (ER^+) MCF-7 and T47D, ER-negative (ER^-) BT-20 breast carcinoma, and human fibroblastic (BJ1) cells (Fig. 2*A*). The highest relative expression was detected for *BCAR1*-1A, 1C, and 1E in all cell lines, whereas 1B, 1B1, and 1D levels were lower. When compared with MCF-10A in T47D and BT-20 cells, significantly higher levels of 1B (4.8- and 4.3-fold, respectively), 1B1 (3.8- and 8.7-fold), 1C (2.1- and 3.2-fold), and 1E (4.4- and 2-fold) were found (Fig. 2*A*, *right panel*).

To further analyze the abundance of the isoforms, real time RT-PCR was conducted on major human organs (breast, colon, stomach, liver, kidney, and rectum) and breast carcinoma and colon carcinoma (Fig. 2*B*). Three samples from each tissue were investigated. As observed for the cell lines, relative expression of *BCAR1*-1A, 1C, and 1E was stronger when compared with 1B, 1B1, and 1D. The variants 1A, 1B1, and 1C were ubiquitously expressed. A more restricted pattern was observed for 1B (detected in only 15 out of 24 samples), 1D (17 out of 24), and 1E (18 out of 24), especially in normal colon and kidney. No trends in expression differences of the variants between normal breast and breast carcinoma were found. Interestingly, isoform 1E was not expressed in normal colon, whereas all colon carcinoma samples had high levels of 1E. These data suggest that the *BCAR1* variants are differentially expressed in human tissues and cell lines.

Reconstitution of $p130^{Cas}$-deficient Fibroblasts with the $p130^{Cas}$ Variants Alters Cell Morphology—The aa sequences encoded by the $p130^{Cas}$ exon 1 variants border the N-terminal

TABLE 3

Database analysis of p130Cas exon 1 variant Cas1B1

Bold and underlined letters indicate amino acid changes compared with *Homo sapiens*.

Species	Cas1B1
Homo sapiens	M PAKPFLSSVLLSWKVLDFSGPGPQGTGQPCSCGHWAEGQGGPPEPAGGP
Pan troglodytes	M PAKPFLSSVLLSWKVLDFSGPGPQGTGQPCSCGHWAEGQGGPPEPAGGP
Macaca mulatta	M P**T**KPFLSS**A**LLSWKVLDFSGPGPQ**E**TGQP**R**SCGH**ET**EGQG**E**PPEPAGG**T**
Physeter catodon	Not found
Bos taurus	Not found
Rattus norvegicus	Not found
Mus musculus	Not found

end of the SH3 domain, which binds to several proteins including FAK (1). To compare the influence of the exon 1 variants on $p130^{Cas}$ phosphorylation, cell morphology, and growth pattern, $p130^{Cas}$-deficient ($Cas^{-/-}$) murine fibroblasts (26) were stably transduced with $p130^{Cas}$ variants Cas1A, 1B, 1B1, 1C, 1D, 1E, or empty vector (ev) (Fig. 3*A*). In addition, we transduced a Cas1Δ deletion construct starting at exon 2 while maintaining the initiating methionine. Expression and size of each variant were confirmed by Western blotting by utilizing low bis-acrylamide gels (32) (Fig. 3*B*). The multiple $p130^{Cas}$ bands are potentially related to posttranslational modifications and/or cleavage products (1).

Equal cell numbers of each transductant were seeded, and the morphology was investigated after 2 (Fig. 3*C*) and 4 days (Fig. 3*D*). Reconstitution of $Cas^{-/-}$ cells with each variant except for Cas1B restored a phenotype characterized by lamellipodia formation accompanied by membrane ruffling (Fig. 3*C*, shown for Cas1A, Cas1B1, and Cas1E). At 2 days in culture, Cas1A and Cas1E transductants exhibited a spindle-like polarized (single leading edge) morphology, whereas cells expressing the longest variant Cas1B1 displayed a rounded cell body and multiple lamellipodia. A different morphology was observed in the Cas1B transductants as most of these cells lacked lamellipodia but instead displayed spike-like protrusions (Fig. 3*C*). Cas1B reconstituted cells primary grew in clusters. In contrast, cells expressing Cas1A or the other variants showed cell scattering (Fig. 3*D*). Phosphorylation of the SD is of major importance for $p130^{Cas}$-mediated cell migration (17, 33). Thus, cells expressing Cas1A, Cas1B, Cas1B1, Cas1C, and Cas1E were harvested after sodium vanadate treatment to accumulate phosphorylated proteins and analyzed by immunoblotting using a mixture of three phospho-Tyr-specific $p130^{Cas}$ abs (Fig. 3*E*). Reconstitution of $Cas^{-/-}$ cells with the variants did not significantly alter SD tyrosine phosphorylation when compared with WT Cas1A. This suggests that alterations in tyrosine phosphorylation on these sites do not correlate with the observed morphological changes.

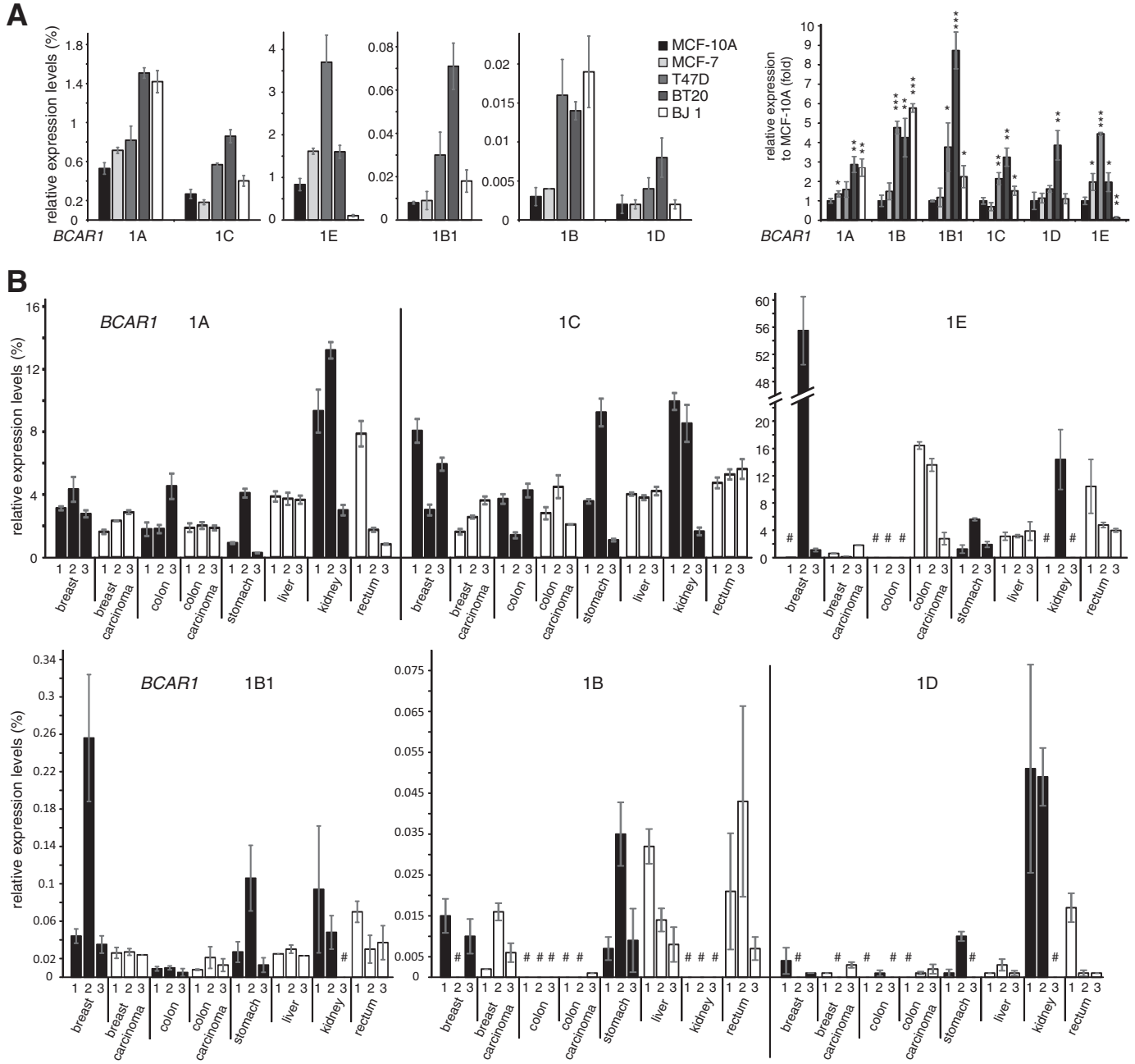

FIGURE 2. ***BCAR1* exon 1 variant mRNA expression in human cells and tissues.** mRNA levels of 1A and the variants 1B, 1B1, 1C, 1D, and 1E were determined by real time RT-PCR. Shown are the results after normalization to GAPDH and SD from triplicate. #, not detected. *A* (*left panels*) and *B*, relative expression levels are shown. Different scales are used. // indicates that parts of the scale were omitted. *A*, *right panel*, average levels relative to the expression in MCF-10A cells (*black bar*, set to 1) are shown. *p* values were calculated using Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ of MCF-10A *versus* the indicated cell line. *Error bars* indicate means ± S.D.

$p130^{Cas}$ Exon 1 Variants with Extended N Termini Show More Efficient Binding to FAK—To test whether the N-terminal variations of $p130^{Cas}$ mediate different interactions with FAK, we generated NT expression constructs of $p130^{Cas}$ (Fig. 4*A*) and performed IP (Fig. 4*B*). The sequences encoding for the different exon 1 variants and the SH3 domain were subcloned in-frame with a 3′ V5 tag. In addition, we generated a NT-1Δ deletion construct. We could not detect NT-1B expression in these studies (not shown). Cell extracts from 293T cells transfected with NT-1A, NT-1C, or the novel variants NT-1B1, NT-1D, NT-1E, or NT-1Δ or ev control were mixed with equal amounts of cell extracts from FAK-transfected 293T cells, immunoprecipitated with V5 abs, and subjected to immunoblotting with FAK (95% of total IP) or V5 (5% of total IP) abs. Equal amounts of NT-1A and the other variants were precipitated (Fig. 4*B*, 5% IP). The longer variants NT-1B1, NT-1D, and NT-1E exhibited considerably more efficient binding to FAK in comparison with all the other isoforms or NT-1Δ (95% IP). Binding of NT-1D and 1E was significantly increased when compared with NT-1B1. NT-1D separated into a faster and slower migrating band (15 and 20 kDa) on an 18% acrylamide gel. Phosphatase treatment greatly reduced the 20-kDa band, indicating that this band is a phosphorylated form of NT-1D (Fig. 4*C*).

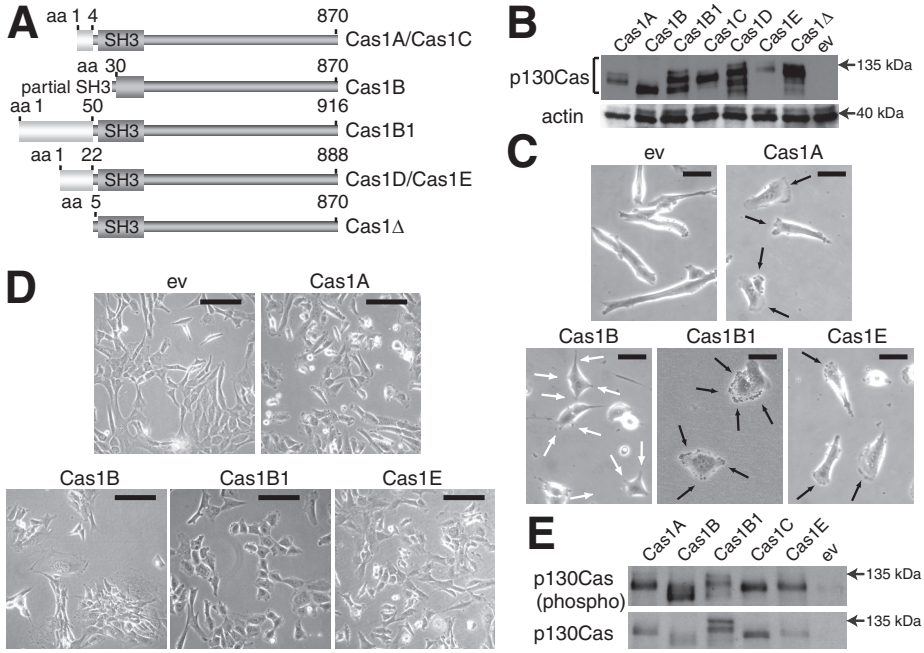

FIGURE 3. **Expression of p130^{Cas} variants alters cell morphology.** Cas$^{-/-}$ fibroblasts were stably transduced with Cas1A, variants 1B, 1B1, 1C, 1D, 1E, or 1Δ, or ev. Results are presented from one experiment out of three performed with similar results. *A*, schematic representation of the full-length p130^{Cas} variants. *B*, whole cell extracts (WCE, 10 μg) were separated on low bis-acrylamide gels and subjected to immunoblotting (IB) with p130^{Cas} and actin abs (control). *C* and *D*, transduced Cas$^{-/-}$ cells (10,000) were seeded in 6-well plates and analyzed by phase contrast microscopy after 2 days (20× magnification; *scale bars*, 50 μm) (*C*) and 4 days (10× magnification; *scale bars*, 250 μm) (*D*). *Black arrows*, lamellipodia and membrane ruffling; *white arrows*, spike-like protrusions. *E*, cells were pretreated with sodium vanadate, and cell extracts were separated on a low bis-acrylamide gel and subjected to IB with total p130^{Cas} abs or a mixture of p130^{Cas}-specific phospho-Tyr (phospho) abs.

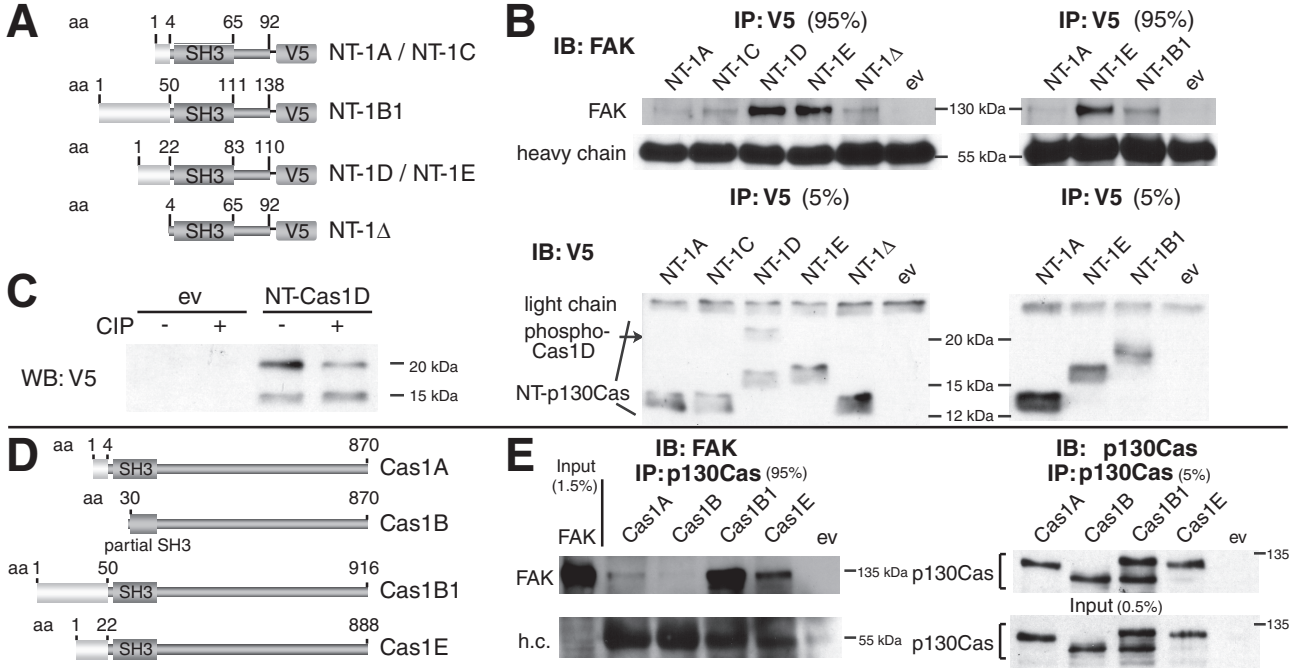

FIGURE 4. **p130^{Cas} variants exhibit differential binding activity to FAK.** *A* and *D*, schematic representations of the constructs: the N-terminal p130^{Cas} variants fused to a V5 tag (*A*) and the full-length p130^{Cas} variants (*D*). *B*, WCE from 293T cells transfected with the N-terminal variants or ev control were mixed with WCE from FAK-transfected 293T cells, immunoprecipitated with V5 abs and probed with FAK abs. *C*, WCE (60 μg) from 293T cells transfected with p130^{Cas} NT-1D or ev control were treated with calf intestinal phosphatase or control-treated and subjected to IB analysis with V5 abs. Results are presented from one representative experiment out of two performed. *WB*, Western blot. *E*, WCE from Cas$^{-/-}$ transductants expressing the full-length variants were mixed with WCE from FAK-transfected 293T cells, immunoprecipitated with p130^{Cas} abs, and probed with FAK abs. *h.c.*, heavy chain. Equal expression of the Cas variants was assessed by IB of 0.5% of WCE. *B* and *E*, 5% of each IP reaction was used to assess equal precipitation of the variants by IB with p130^{Cas} abs. Results are presented from one experiment out of three performed with similar results.

To elucidate whether altered binding to FAK can also be seen with the full-length p130^{Cas} variants, the interaction of Cas1A, the novel Cas1B, Cas1B1, and Cas1E with FAK was investigated by IP (Fig. 4, *D* and *E*). Whole cell extracts from Cas$^{-/-}$ transductants were mixed with cell extracts from FAK-transfected 293T cells, immunoprecipitated with p130^{Cas} abs, and exam-

ined for FAK or $p130^{Cas}$ expression (Fig. 4*E*). Equal amounts of each full-length isoform were used as input and precipitated (Fig. 4*E*, *right panel*). Importantly, almost no association with FAK was detected for truncated Cas1B (Fig. 4*E*, *left panel*). Cas1E showed an increased binding to FAK when compared with Cas1A. Contrary to the N-terminal construct results (Fig. 4*B*), binding of full-length Cas1B1 was significantly enhanced in comparison with Cas1E (Fig. 4*E*). Together, these results indicate that the longer N-terminal extensions present in Cas1B1, Cas1D, and Cas1E increase the binding of the $p130^{Cas}$ SH3 domain to FAK.

$p130^{Cas}$ Exon 1 Variants Influence the Migratory and Invasive Potential of $Cas^{-/-}$ Fibroblasts—To investigate whether increased binding of the $p130^{Cas}$ variants to FAK alters the migratory and invasive behavior, serum-stimulated migration and invasion assays were performed with $Cas^{-/-}$ cells expressing Cas1A, 1B, 1B1, or 1E (Fig. 5). Cas1A reconstituted cells were used as reference cells. Expression of the truncated variant Cas1B significantly reduced migration by 73% ($p < 0.01$) and reduced invasion by 75% ($p < 0.05$) when compared with WT Cas1A. Reconstitution with the longest variant Cas1B1 increased migration by 39% ($p < 0.05$), whereas the invasion rate was not altered. Cas1E expression did not influence migration, but invasion was reduced by 37% ($p < 0.01$).

Taken together these results indicate that the $p130^{Cas}$ exon 1 variants display altered functional properties. Importantly, the truncated variant Cas1B, lacking parts of the SH3 domain, and Cas1B1, comprising the longest N-terminal extension, exerted greatly reduced and increased functionality, respectively.

DISCUSSION

Here we identified four novel $p130^{Cas}$/*BCAR1* mRNA variants encoded by alternative first exons, resulting in functionally different protein isoforms. The presence of two alternative first exon variants of $p130^{Cas}$ was first identified in mice (15) and recently also by us in humans (10). Database searches and detailed analysis of *BCAR1* revealed four additional mRNAs that are transcribed from alternative first exons (1B, 1B1, 1D, and 1E). Exons 1A and 1C correspond to the previously described mouse variants (15). Our studies show that the *BCAR1* isoform mRNAs are commonly co-expressed in cell lines and in major human organs. The more widely distributed *BCAR1* mRNAs are *BCAR1*-1A, 1B1, and 1C, whereas expression of the other mRNAs is more restricted. Highest relative expression was observed for *BCAR1*-1A, 1C, and 1E, whereas lower levels were found for the other variants.

These results and the database analysis for *BCAR1* suggest the utilization of multiple promoters to drive the expression of the exon 1 variants.[4] Alternative promoter usage can result in the expression of different non-coding first exons (34, 35) and also in the expression of functionally distinct N-terminal protein variants (36, 37). Most of the *BCAR1* exon 1 variants belong to the latter. They encode for aa sequences flanking the N-terminal end of the SH3 domain. The $p130^{Cas}$ SH3 domain is important for binding to FAK and several other proteins (1, 3) and the translocation of $p130^{Cas}$ to FAs and leading edges of the

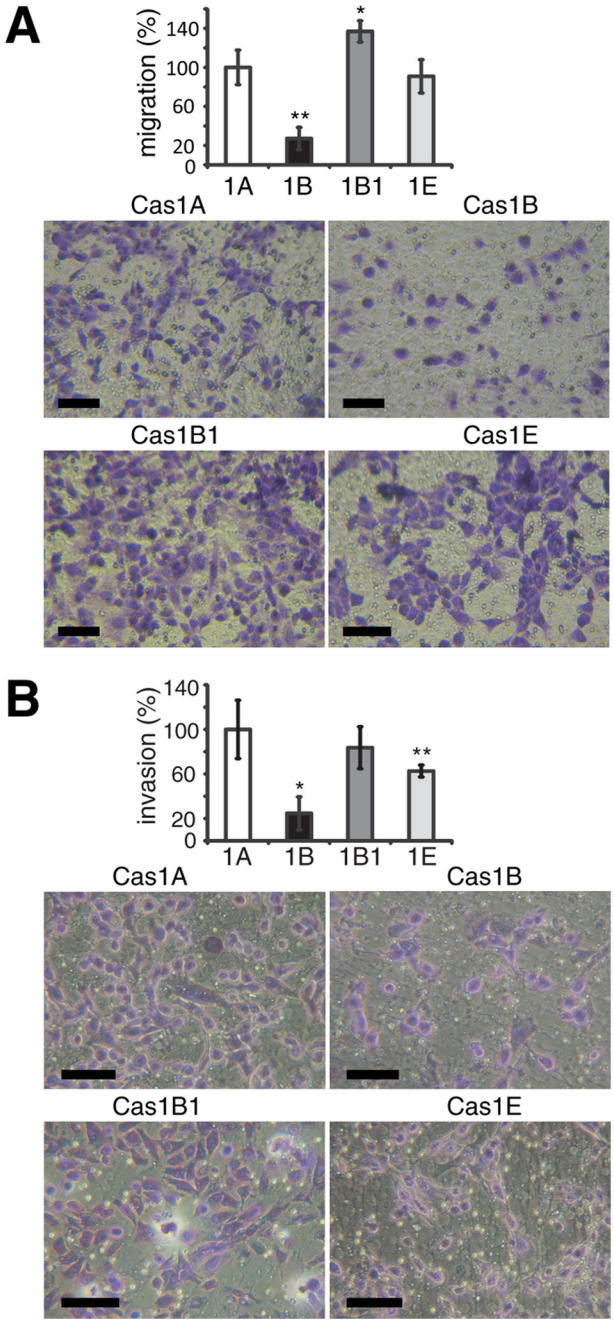

FIGURE 5. **$p130^{Cas}$ variants alter the migratory and invasive potential.** *A* and *B*, $Cas^{-/-}$ cells stably and constitutively expressing the indicated constructs were subjected to serum-stimulated migration assay for 6 h (*A*) and invasion assay for 22 h (*B*) as described. *Upper panels*, shown are the percentage of migration/invasion (Cas1A was set to 100%) and S.D. of triplicate from three independent experiments. *, $p < 0.05$, and **, $p < 0.01$ of Cas1A *versus* the indicated variant. *p* values were calculated using Student's *t* test. *Lower panels*, shown are representative images from three independent experiments (10× magnification; *scale bars*, 200 μm).

cell (17, 19, 38). Exon 1B is non-coding, producing a truncated $p130^{Cas}$ variant (Cas1B) that lacks a portion of the SH3 domain. The longer protein variants Cas1B1, 1D, and 1E showed significantly more efficient binding to FAK when compared with the shorter Cas1A (WT) and 1C. How this occurs is not clear, although data suggest that these additional sequences positively regulate the interaction of $p130^{Cas}$ with FAK. All of the

[4] J. Kumbrink and K. H. Kirsch, unpublished data.

longer N-terminal extensions are likely unstructured[5] but may provide additional binding surfaces for FAK. This could result in increased FAK binding to $p130^{Cas}$ or a more stable complex formation as described for myosin-2 (39) and phospholipase Cg-1 (40). Moreover, our results suggest that additional domains in the C-terminal part support efficient binding of the longest variant Cas1B1 to FAK. The C-terminal domain contains a second focal adhesion targeting domain (41), which may contribute to this observation. The stronger binding could also be mediated by posttranslational modifications because the Cas1D N terminus is in part modified by phosphorylation.

Reduced association with FAK was observed for Cas1B, likely due to its truncated SH3 domain. Most of the Cas1B-expressing cells lacked lamellipodia and did not form a distinct leading edge. These cells grew in tight clusters, whereas cells expressing the most studied Cas1A or the other variants showed a high degree of cell scattering. The partial deletion of the SH3 domain in Cas1B corroborates the importance of the SH3 domain in establishing a polarized phenotype associated with cell motility and FA formation (1, 42, 43). Importantly, reconstitution of Cas1B in $Cas^{-/-}$ cells significantly reduced cell migration and invasion when compared with Cas1A cells. These characteristics suggest that the novel Cas1B variant is a naturally occurring $p130^{Cas}$ isoform with greatly decreased functionality. Deletion of the SH3 domain has been shown to significantly impair the localization of $p130^{Cas}$ to FAs, accompanied by a substantial reduction of tyrosine phosphorylation within the SD (17). Phosphorylation of the SD is of major importance for $p130^{Cas}$-mediated cell migration (33). Unexpectedly, cells expressing the truncated Cas1B show no alteration in tyrosine phosphorylation of the SD. Thus, the phosphorylated SD of Cas1B may act as a decoy for common $p130^{Cas}$ SD-binding proteins without mediating SH3 domain function.

In contrast, the longest variant Cas1B1 exhibited greatly enhanced functionality. This is indicated by its most efficient FAK binding among all variants and a significantly enhanced potential to drive cell migration. The SD tyrosine phosphorylation of Cas1B1 was not influenced. However, the N-terminal extension of 50 aa contains several potential additional interaction motifs, which may recruit other factors and/or stabilize FAK binding to enhance and potentially alter the functionality of Cas1B1. Our studies have identified novel $p130^{Cas}$ variants that exhibit functional differences including naturally occurring isoforms with greatly diminished and increased functionality.

How the isoforms that are often co-expressed in various organs affect $p130^{Cas}$ function (for example, the truncated Cas1B by acting dominant negative or single variants by activating distinct cellular programs) is not clear. Interestingly, Cas1E was not detected in normal colon samples, whereas all colon carcinomas had high levels. However, the low sample number does not allow for any statistical correlation but allows for a trend. The higher expression levels of Cas1A, 1C, and 1E may imply a greater importance of these isoforms than of the other variants. Nevertheless, we have recently shown that Cas1A and 1C expression levels can be influenced by external stimuli (10). Analysis of the putative promoter regions of the other isoforms also identified potential binding sites for multiple inducible transcription factors.[4] This suggests that the expression levels of each variant may be influenced by the same or even different stimuli/pathways. Future studies aimed to characterize these variants could potentially unravel new mechanisms by which the $p130^{Cas}$ variants contribute to the regulation of various cellular programs and how their regulation may contribute to the development of malignancies.

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